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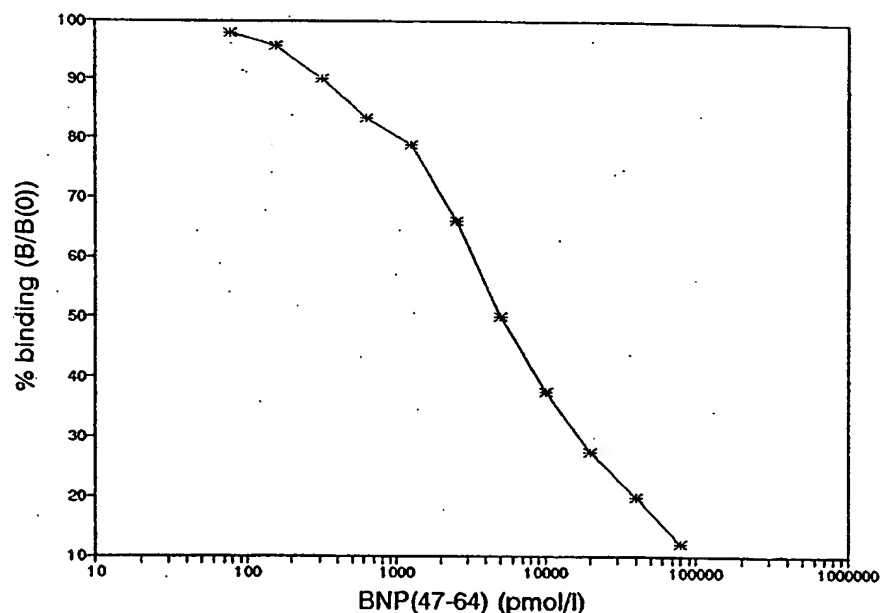
(57) Abstract

The present invention provides antibodies for use in a method of immunoassay being antibodies specific to the polypeptide comprising amino acids 1-76 of N-terminal brain natriuretic factor BNP(1-76). Also provided are methods and kits for the diagnosis or prognosis of conditions in which BNP(1-76) is a diagnostic or predictive indicator e.g. heart failure or hypervolaemia.

BNP ANTIBODY AND IMMUNOASSAY USING IT

This invention relates to the N-terminal section of
Brain Natriuretic Peptide Prohormone, BNP(1-76) and the
use of antibodies against this in immunoassays in
biological fluids for the purpose of biological research
and medical diagnosis, for example of heart failure or
hypervolaemia.

Heart failure is a common clinical syndrome
especially among elderly people. Population surveys
indicate that the condition affects about 2% of the
total population in the western world. The syndrome
usually presents itself with an insidious onset with
unspecific symptoms such as dyspnea on exertion, fatigue
and peripheral oedemas. To establish the diagnosis the
physician usually must either rely on his clinical
experience or refer the patient to a cardiological
center for echocardiography, radionuclide scanning,
exercise testing or catheterization.

Heart disease represents a significant drain on
health resources in many major countries, and whilst an
early diagnosis may help in controlling the condition
and preventing rapid progression to severe heart
failure, it would obviously be preferable to be able to
identify those patients in which heart failure is likely
to occur before it actually does so, ie. to prognose
rather than diagnose.

Unfortunately, there are at present no completely
satisfactory methods for predicting the likelihood of
heart failure. Problems frequently observed with such
methods are insufficient accuracy and sensitivity, and
the disadvantages of the necessity for expensive
equipment requiring specially trained personnel (eg. in
echocardiography). A need therefore exists for a simple
method of accurately and sensitively, not only
diagnosing, but also predicting the likelihood of onset

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of heart failure.

Whilst heart failure can be defined as a
symptomatic state ie. an overt disease or syndrome,
patients may frequently pass through a state of
asymptomatic cardiac dysfunction ie. a sub-clinical
condition without overt symptoms, before heart failure
manifests itself. However, we have now found that not
all patients having cardiac dysfunction go on to develop
severe heart failure, and that the risk of heart failure
for some such people is much greater than for others.
To be able to identify those people at particular risk
of developing heart failure in order that they may be
caught and treated before heart failure occurs would be
of great clinical importance; at the moment existing
treatments eg. ACE inhibitors are very expensive and it
is not cost-effective for everyone to be treated to try
to prevent the onset of heart failure.

Brain Natriuretic Peptide (BNP) is a polypeptide
originally isolated from porcine brain by T. Sudoh and
coworkers (Nature 1988; 332: 78-81). After cloning and
sequence analysis of cDNA coding for the peptide (T.
Sudoh BBRC 1989; 159: 1427-34) human BNP was shown to be
produced in the human heart. Human Brain Natriuretic
Peptide is believed to be produced in cardiac myocytes
as a prohormone (proBNP or BNP(1-108)). proBNP consists
of 108 amino acids and is split, before or during
secretion, at amino acids Arg76 -- Ser77 into BNP and
the N-terminal part of the prohormone, BNP(1-76), that
is the peptide consisting of the first 76 amino acids
from the N-terminal of proBNP.

The BNP(77-108) plasma concentration is increased
in patients suffering from heart disease leading to
heart failure. The cardiac monocytes secrete another
factor, namely atrial natriuretic factor (ANF) but the
secretory response to heart failure or incipient heart
failure seems to be much larger in the BNP system
compared to the ANF system (Mukoyama et al, J Clin

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Invest 1991; 87: 1402-12).

The present invention is based on the concept that BNP(1-76), due to a long half-life as compared with BNP hormone itself and high initial concentration, is a particularly good diagnostic indicator or predictor of heart disease and also of hypervolaemia.

BNP(1-76) may thus be used to provide the basis of either a diagnostic or a prognostic test for heart failure, primarily in the biosynthesis of antibodies for use in such a test but also as competing antigen in competitive binding immunoassays. For such use in making antibodies BNP(1-76) or a fragment thereof may advantageously be conjugated to an immunogenic protein or peptide such as PPD, a protein derivative of tuberculin, Keyhole Limpet Haemocyanin or bovine serum albumin.

Thus BNP(1-76) or an antigenic fragment thereof or polypeptide extension thereof having at least one antigenic epitope of BNP(1-76), conjugated to one or more immunogenic polypeptides, constitutes one aspect of the present invention; these polypeptides may be used to make either polyclonal or monoclonal antibodies specific to BNP(1-76). Such monoclonal and polyclonal antibodies constitute two further aspects of the invention.

According to a still further aspect of the invention we provide a method of immunoassay for BNP(1-76) or a fragment thereof or polypeptide extension thereof wherein the primary binding partner therefor is a monoclonal or polyclonal antibody according to the invention. Methods of immunoassay are of course well known in the art eg. RIA, ELISA, fluorescence immunoassay (FIA) or dry chemistry test strip immunoassays. Such an immunoassay will, in general, use a monoclonal or polyclonal antibody according to the invention in immobilised form, eg. on microtitre plates, membranes or beads, to isolate the target BNP(1-76) compound. In a sandwich assay, the bound antigen may be

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labelled using additional soluble antibody according to
the invention, which may be monoclonal or polyclonal and
which may either carry a label or, more conveniently,
may itself be labelled subsequently by reaction with a
secondary antibody carrying a label.

Thus, if the primary antibody according to the
invention is raised in mice or rabbits, the labelled
secondary antibody may be an anti-mouse or anti-rabbit
antibody.

Suitable labels include radionucleides, fluorescent
substances eg. europium based fluorogens, enzymes, for
example as used in ELISA systems employing automated
hybrid methods or dyes or coloured particles such as
colloidal gold.

Alternatively, a competitive binding assay may be
used, wherein a known quantity of labelled BNP(1-76), or
fragment or extension thereof, is added to the analyte
solution and contacted with a limited quantity of the
immobilised monoclonal or polyclonal antibody, whereby
the amount of labelled antigen which is immobilised is
inversely proportional to the amount of target antigen
present in the analyte.

The invention thus extends to labelled forms of
BNP(1-76) or fragments or polypeptide extensions thereof
and to labelled forms of the antibodies of the
invention.

The invention also comprises a kit for immunoassay
of BNP(1-76) or a fragment or polypeptide extension
thereof comprising at least:

(a)   a monoclonal or polyclonal antibody according
to the invention in immobilised form and, optionally;

(b)   a labelled sample of BNP(1-76) or a fragment
or polypeptide extension thereof;

(c)   said monoclonal or polyclonal antibody in non-
immobilised form;

(d)   a labelled secondary antibody specific to said
antibody (c).

Such an immunoassay and kit may be used in research
into related biological systems as well as for diagnosis
or prognosis of conditions wherein the BNP(1-76) level
in body fluids is a diagnostic or predictive indicator.

The invention also comprises a method of diagnosis
or prognosis of a condition in which the concentration
of BNP(1-76) or a fragment or polypeptide extension
thereof is a diagnostic or predictive indicator, wherein
a body fluid of a patient is subjected in vitro to
immunoassay to detect or assay the presence or quantity
therein of BNP(1-76).

We have recently found that another natriuretic
factor namely pro-ANF, and in particular N-terminal pro-
ANF, can serve as an indicator of risk of heart failure
in patients lacking overt symptoms of heart failure.
The level of pro-ANF in body fluids can be directly
related to the risk of heart failure, predominantly
related to increased atrial pressure. In contrast,
BNP(1-76) is predominantly an indication of a heart
condition related to increased ventricular pressure.
BNP(1-76) as a fragment or polypeptide extension thereof
can also be used to assess risk of heart failure in
addition to its use in diagnosis of actual heart
failure. Furthermore, assay of both N-terminal pro-ANF
and BNP(1-76) in body fluids can assist in determining
whether atrial or ventricular pressure is concerned.

Thus, the immunoassay can be used in the monitoring
of heart failure treatment. Such treatment is aimed at
reducing the hypervolemia and excessive vasoconstriction
seen in heart failure by the administration of diuretics
and vasodilators. By decreasing the pressure in the
cardiac chambers such treatment will lower cardiac
production of BNP(1-76). The resultant decrease in
plasma BNP(1-76) concentration serve to inform the
physician of a significant drug effect. On the
contrary, an increase in plasma BNP(1-76) indicates that
an adjustment of dosage might be necessary.

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Although less well documented at this time BNP(1-76) may also be used as a diagnostic tool in the diagnosis of hypervolemia without heart failure. The immunoassay therefore has potential use also in the inhospital intensive care setting where monitoring of volume status is essential.

The body fluid on which the immunoassay is performed may be any body fluid in which the BNP(1-76) is located, but conveniently will be plasma or serum. In some cases it may be convenient to extract the peptide, or otherwise treat the sample prior to assay.

The BNP(1-76) peptide or an antigenic or immunogenic fragment thereof may be produced by synthesis from its constituent amino acids or by assembly of pre-synthesised blocks of amino acids using techniques well known in the art. Where labelled material is required, the label may be introduced by standard techniques.

For the purpose of raising monoclonal or polyclonal antibodies, the BNP(1-76) or fragment thereof may be conjugated to an immunogenic protein or peptide, for example PPD, a protein derivative of tuberculin, eg. using 1-ethyl-3-(3-dimethyl-aminopropyl)carbodiimide according to the method of Staros et al (Analyte Biochem 1986; 156: 220-222).

The antibodies of the invention may be made by injecting a host animal, eg. a mouse or rabbit, with the BNP antigen of the invention, advantageously a conjugate with an immunogenic protein as described above, to provide either a serum containing polyclonal antibodies or spleen cells for conversion to hybridomas or immortalised cell lines producing monoclonal antibodies.

The following Examples are given by way of illustration only with reference to the accompanying drawing in which:

Figure 1 shows a standard curve for immunoassay for BNP(1-76) using synthetic BNP(47-64) as immunogen,

standard and tracer, and polyclonal rabbit antibody.
(Abscissa shows BNP(47-64) pmol/l; ordinate shows %
binding (B/B(O))).

Example 1

Production of monoclonal antibody against BNP(1-76)

1)   Conjugation

Three synthesized fragments of BNP(1-76): BNP(1-
21), BNP(22-46) and BNP(47-64) were acquired from
Peninsula laboratories and conjugated to PPD (protein
derivative of tuberculin) according to Staros et al
(Analyt Biochem 1986; 156: 220-222).

2)   Immunization

Balb C mice, preimmunized with BCG antigen were
utilized.   The mice received a 50 microgram mixture of
the three conjugates in 200 µl of Freunds incomplete
adjuvant.   The mixture was given in 2 x 200 µl
injections on 2 occasions 2 weeks apart.   2 weeks after
the last injection 50 µg of conjugate mixture in saline
was injected intraperitoneally.

3)   Fusion

3 days after intraperitoneal immunization mouse
splenic cells were fused with SP 2/0 myeloma cells and
the resultant hybridomas selected in HAT medium.   The
suspension of hybridomas was distributed in 960 wells in
Dulbeccos medium enriched with 10% human endothelial
cell supernatant.

4)   Screening

Method 1

Costar microtiter plates were coated with a mixture
of the synthetic BNP peptide sequences (0.5 µg/ml).

Supernatants were then added and binding of antibody
from supernatants was screened by ELISA through the
addition of anti mouse IgG conjugated to horseradish
peroxidase enzyme followed by substrate solution (OPD).

Method 2

An alternative method of screening is to coat
Greiner microtiter plates with goat anti mouse IgG (1.0
μg/ml). Supernatants are then added and incubated.
Biotinylated synthetic BNP peptide sequences are added
and the ability of supernatants to bind peptide are
screened by ELISA through the addition of streptavidin-
conjugated horseradish peroxidase enzyme followed by
substrate solution (OPD).

5)   Cloning

Hybridomas producing antibodies to the peptide
mixture were cloned and subcloned in two runs. Clone
1C7 was shown to react with peptide sequence BNP(47-64).
This clone was grown and the supernatant utilised in
immunoassay for BNP(1-76).

Example 2

Immunoassay for BNP(1-76)

The 1C7 antibody can be utilised in various types
of immunoassays for BNP(1-76). These include

    a)   Radioimmunoassay (RIA)
    b)   Europium Fluorescence immunoassays (FIA)
    c)   Enzyme linked immunosorbent assays
          (ELISA) including automated hybrid methods
          running on micro titer plates or membranes
    d)   Various dry-chemistry test strip immunoassays

The following is an example of a sandwich ELISA.

Costar microtiter plates are precoated with the 1C7 antibody. Sample or standard is added to the wells and after 2 hours of incubation the wells are washed and a secondary antibody (polyclonal or monoclonal) towards BNP(1-76) is added. Again after 2 hours a horseradish peroxidase-labelled anti mouse (rabbit) antibody is supplied and finally after the addition of O-phenylene-diamine substrate the colour is read in a platereader.

Example 3

Immunoassay for BNP(1-76) utilizing polyclonal rabbit antibody

A synthetic peptide subsequence of BNP(1-76), in this case BNP(47-64), was conjugated to PPD according to Staros et al., (Supra). Rabbits were BCG vaccinated and then repeatedly immunized with the conjugated peptide.

Iodination (^{125}I) of synthetic BNP(47-64), with a tyrosine group added at the N-terminal end, was done by the chloramine-T method as follows:

Chloramine-T Method

1)   5 μg of the synthetic peptide was reconstituted with 20 μl sodiumphosphate buffer (0.25 M, pH 7.5).

2)   Approximately 5 μl of ^{125}-I was added (0.5 mCi).

3)   5 μl of chloramine-T (1 mg/ml) was added and incubated for 45 seconds.

4)   5 μl of sodiummetabisulphite (1 mg/ml) was added and incubated for 45 seconds.

5)   The mixture was then fractionated on a column with Sephadex G10.

6)   The fractions were counted with a gamma-counter,
     and the fraction/fractions with highest counts per
     minute (cpm) were selected as tracer for use in the
     RIA methods.

     Sample or standards (BNP[47-64]), together with
tracer (iodinated BNP(47-64)) and polyclonal antibody
from rabbit serum, are mixed in polystyrene assay tubes.
After 48 hours of incubation at 4°C, normal serum from
rabbit, and goat anti-rabbit IgG are added. After 2
hours of incubation, polyethyleneglycol (PEG) is added
and the samples are centrifuged. The supernatant is
removed and the counts per minute (cpm) in precipitate
are measured with a gammacounter. An example of a
standard curve obtained by this type of assay is shown
in Figure 1.

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<u>Claims</u>

1.   Antibodies for use in a method of immunoassay being
antibodies specific to the polypeptide comprising amino
acids 1-76 of N-terminal brain natriuretic factor BNP(1-
76).

2.   Antibodies as claimed in claim 1 being monoclonal
antibodies.

3.   Antibodies as claimed in claim 1 being polyclonal
antibodies.

4.   Antibodies as claimed in any of claims 1 to 3
carrying a label.

5.   Antibodies as claimed in claim 1 in which the label
is a radionuclide, a fluorescent substance, an enzyme, a
dye or coloured particles.

6.   Antibodies as claimed in any of claims 1 to 5 in
immobilised form.

7.   A method of immunoassay for BNP(1-76) or a fragment
thereof or polypeptide extension thereof wherein the
primary binding partner therefor is a monoclonal or
polyclonal antibody according to any one of claims 1 to
6.

8.   A method as claimed in claim 7 in which the
antibody is immobilised as microtitre plates, membranes
or beads.

9.   A method as claimed in claim 8 in which a secondary
antibody against BNP(1-76) is used in a sandwich assay
and is labelled before or after reaction with BNP(1-76).

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10. A method as claimed in claim 9 in which a known
quantity of labelled BNP(1-76) or a fragment or
polypeptide extension thereof is added to an analyte
solution and contacted with a limited quantity of
immobilised antibody against BNP(1-76) to provide a
competitive binding assay.

11. Labelled BNP(1-76) or a fragment or polypeptide
extension thereof.

12. BNP(1-76) or an antigenic fragment thereof or
polypeptide extension thereof having at least one
antigenic epitope of BNP(1-76), conjugated to one or
more immunogenic polypeptides.

13. A kit for immunoassay of BNP(1-76) or a fragment or
polypeptide extension thereof comprising at least:
    (a) a monoclonal or polyclonal antibody according
to the invention in immobilised form and, optionally;
    (b) a labelled sample of BNP(1-76) or a fragment
or polypeptide extension thereof;
    (c) said monoclonal or polyclonal antibody in non-
immobilised form;
    (d) a labelled secondary antibody specific to said
antibody (c).

14. A method of diagnosis or prognosis of a condition
in which the concentration of BNP(1-76) or a fragment or
polypeptide extension thereof is a diagnostic or
predictive indicator, wherein a body fluid of a patient
is subjected in vitro to immunoassay to detect or assay
the presence or quantity therein of BNP(1-76).

15. A method for the production of an antibody as
claimed in claim 1 wherein BNP(1-76) or a fragment or
polypeptide extension thereof, if necessary conjugated
to an immunogenic protein or polypeptide, is injected

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into a host animal to provide a serum containing a
polyclonal antibody or spleen cells which are
subsequently converted to hybridomas or immortalised
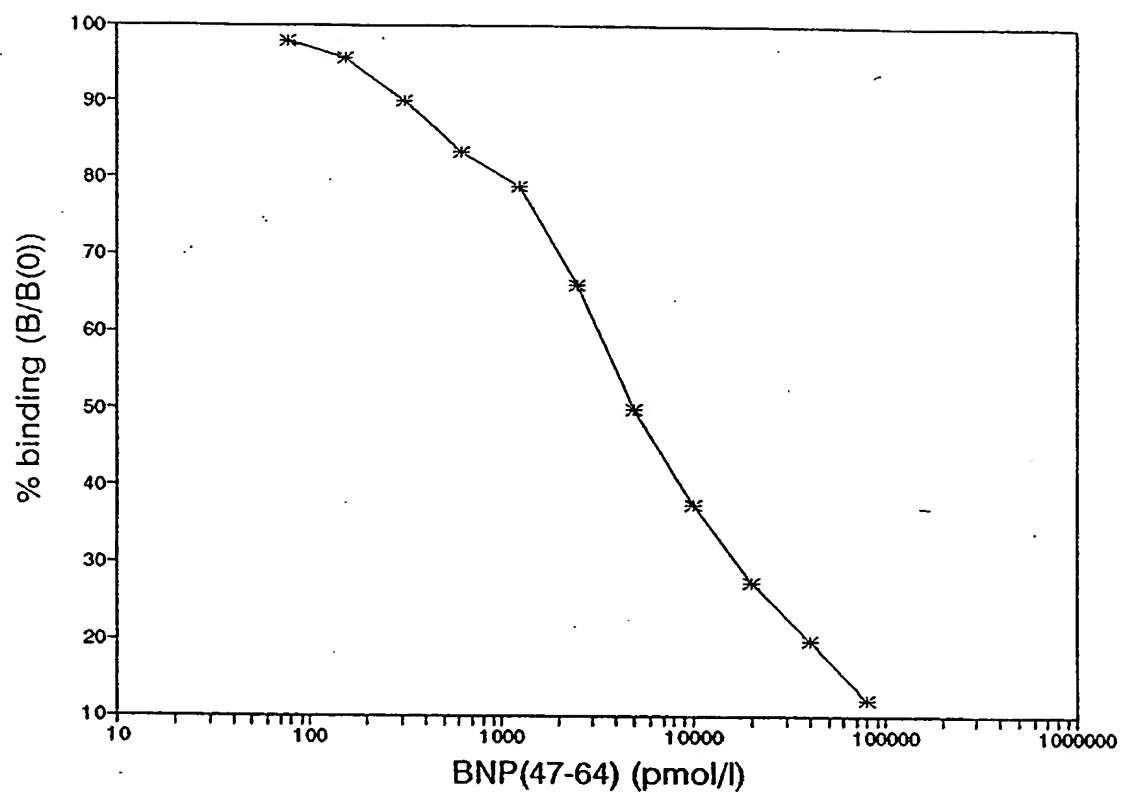cell lines producing monoclonal antibodies.

Figure 1

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all)
According to International Patent Classification (IPC) or to both National Classification and IPC

Int.Cl. 5 C07K15/28; C07K7/00; C12P21/08; G01N33/53
G01N33/577; G01N33/68

II. FIELDS SEARCHED

Minimum Documentation Searched[7]

Classification System	Classification Symbols
Int.Cl. 5	C07K ; C12P ; G01N

Documentation Searched other than Minimum Documentation
to the Extent that such Documents are Included in the Fields Searched[8]

III. DOCUMENTS CONSIDERED TO BE RELEVANT[9]

Category[o]	Citation of Document,[11] with indication, where appropriate, of the relevant passages[12]	Relevant to Claim No.[13]
X	JAPANESE PATENTS ABSTRACTS (UNEXAMINED) Section Ch, Week 9207, Derwent Publications Ltd., London, GB; Class CH, AN 92-053618 & JP,A,3 297 392 (SHIONOGI KK) 27 December 1991 see abstract	1-10
X	CHEMICAL ABSTRACTS, vol. 114, no. 25, 24 June 1991, Columbus, Ohio, US; abstract no. 240736k, TOGASHI, KAZUYOSHI ET AL. 'A specific and highly sensitive radioimmunoassay of human brain natriuretic peptide' page 108 ;column L ; & RHINSHO BYORI vol. 39, no. 3, 1991, JAPAN; pages 283 - 288 see abstract	1-10

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° Special categories of cited documents :[10]

"A" document defining the general state of the art which is not considered to be of particular relevance

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"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report
25 AUGUST 1993	1 4 -09- 1993

International Searching Authority	Signature of Authorized Officer
EUROPEAN PATENT OFFICE	HORNIG H.

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)		
Category*	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No.
A	J. CLIN. INVEST. vol. 87, no. 4, April 1991, AM. SOC. CLIN. INVEST., pages 1402 - 1412 M. MUKOYAMA ET AL. 'Brain natriuretic peptide as a novel cardiac hormone in humans' cited in the application see page 1404, left column, paragraph 6 - page 1408, right column, paragraph 2	1-15
A	EP,A,0 385 476 (DAIICHI PURE CHEMICALS CO.) 5 September 1990 see page 2, line 45 - page 3, line 3; claims 1-9	1-15

ANNEX TO THE INTERNATIONAL SEARCH REPORT
ON INTERNATIONAL PATENT APPLICATION NO.

GB   9301173
SA   74642

This annex lists the patent family members relating to the patent documents cited in the above-mentioned international search report.
The members are as contained in the European Patent Office EDP file on
The European Patent Office is in no way liable for these particulars which are merely given for the purpose of information.   25/08/93

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
EP-A-0385476	05-09-90	JP-A- 2231082	13-09-90
		JP-A- 2237999	20-09-90